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Isotopic methods for non-destructive assessment of carbon dynamics in shrublands under long-term climate change manipulation

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Running head: Isotope techniques for ecosystem C science

Tweet: The pros and cons of carbon assessment methods using isotopes across climate change experiments in shrublands.

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Summary

1. Long-term climate change experiments are extremely valuable for studying ecosystem responses to environmental change. Examination of the vegetation and the soil should be non-destructive to guarantee long-term research. In this paper, we review novel field methods using isotope techniques for assessing carbon dynamics in the plant-soil-air continuum, based on recent field experience and examples from a European climate change manipulation network.

2. Eight European semi-natural shrubland ecosystems were exposed to controlled warming and drought manipulations. One field site was additionally exposed to elevated atmospheric CO₂. We evaluate the isotope methods that were used across the network to evaluate carbon fluxes and ecosystem responses: 1) analysis of the naturally rare isotopes of carbon (¹³C and ¹⁴C) and nitrogen (¹⁵N); 2) tracing changes in isotopic signatures in ecosystem compartments *in-situ*, by using pulse labelling with ¹³CO₂, soil injections of ¹³C- and ¹⁵N-enriched substrates, or continuous labelling with ¹³C-depleted CO₂ by Free Air Carbon dioxide Enrichment (FACE); and 3) manipulation and tracing the isotopic composition of soil substrates (¹⁴C) in lab-based studies.

3. Questions related to long-term carbon turnover processes were investigated by natural ¹⁴C signals, specifically ¹⁴C signature of soil respiration gave insights into the decomposition of old soil carbon sources. Contrastingly, the stable isotopes ¹³C and ¹⁵N were used for shorter-term processes, as the residence time in a certain compartment of the stable isotope label signal is limited. ¹³C-labelling techniques exert a minimal physical disturbance, however, the dilution of the applied isotopic signal can be challenging, and the contamination of the field site with released excess ¹³C can be a problem for subsequent natural abundance (¹⁴C and ¹³C) or label studies.

4. Based on the experience with the experimental work, we provide recommendations for the application of the reviewed methods to study carbon fluxes in the plant-soil-air continuum in long-term, large-scale climate change experiments.

Key-words: warming; drought; bomb-C; FACE; pulse-labelling; stable isotopes; ¹⁴C

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Introduction

Global climate change scenarios predict that increased greenhouse gas (e.g. CO_2 , CH_4 and N_2O) concentrations in the atmosphere will alter the periodicity and magnitude of drought events and will increase mean global temperatures by approximately $0.2\text{ }^\circ\text{C}$ per decade (IPCC 2013). For the European continent this will manifest as drier summers in the South and increased precipitation in the North (IPCC 2013). Elucidating the consequences of such atmospheric changes for biogenic carbon fluxes is one of the main challenges for the scientific

community. Some models have predicted a positive feedback to climate change, resulting from higher increases in respiratory fluxes from ecosystems (e.g. carbon release through soil respiration) than in net primary productivity, which would lead to further increases in atmospheric CO₂ (Friedlingstein et al. 2006; Denman et al. 2007). To assess the likelihood of positive feedback, experimental studies that analyse the long-term adaptations of ecosystem carbon fluxes to climate change are critically needed. However, climate change experiments are often conducted at short or medium time scales due to funding constraints, or due to the limited life-span of the experimental plots, as repeated removal of samples often leads to disturbances and experimental artefacts in the studied system. Hence, there is a necessity for the maintenance of long-term experiments using non-destructive methods.

Carbon fluxes through the plant-soil-air continuum play a central role in soil carbon cycling. Drought and warming alter the quantity and composition of carbon inputs to the soil by changing plant available carbon and nitrogen sources (de Graaff et al. 2007; Leakey et al. 2009). Microbial growth in soil is generally constrained by available carbon, therefore qualitative and quantitative changes in rhizodeposition are likely to alter the activity of heterotrophic microorganisms and the rates of soil organic matter (SOM) mineralisation (Zak et al. 2000; Phillips et al. 2006). Consequently, aboveground to belowground fluxes might largely determine carbon emissions from ecosystems under the different climate change scenarios (Chapin et al. 2009).

Stable carbon isotope studies can give important insights into carbon fluxes through the plant-soil-air continuum with the minimal disturbance to the system. The isotopic carbon composition of compartments in this continuum is a result of the different isotope fractionation processes along the pathway from CO₂ fixation by plants to carbon allocation to soil (reviewed in Brüggemann et al. 2011). Thus, the analysis of the natural abundance of carbon isotopes in these compartments can give information about some processes related to photosynthesis and carbon losses through plant or soil respiration (Brüggemann et al. 2011). In addition, *in-situ* pulse labelling with the heavy stable carbon isotope (¹³C) is a powerful tool to analyse short-term dynamics of carbon allocation to the soil with high resolution (Högberg et al. 2008; Epron et al. 2012; Reinsch & Ambus 2013). The recent development of techniques for ¹³C analyses in specific compounds such as phospholipid fatty acids (PLFAs), amino-sugars, RNA and DNA, constitutes a remarkable advance in the studying of carbon cycling. These analyses allow for the examination of rhizodeposit utilisation by microbes or trophic interactions between functional groups (Ostle et al. 2003; Jin & Evans 2010; Ruess & Chamberlain 2010). The

application of these isotopic methods can therefore provide unique information about aboveground-belowground linkages and their alterations in response to climate changes.

In order to investigate long-term effects of climate change on shrubland ecosystems, an experimental network was established across Europe (the INCREASE network). Studying the response of shrublands to climate change is important, since they are representative ecosystems in Mediterranean and North European countries, where they play an important ecological role in preserving biodiversity (Verdú 2000; Wessel et al. 2004). In addition, land area covered by shrublands has dramatically decreased in temperate Europe during the past century, due to land use changes, increased pollution and eutrophication, and climate change (Fagúndez 2013). In Mediterranean regions, however, shrublands have increased their extension due to land abandonment (Fagúndez 2013).

Within the climate change network, common non-destructive methods were used across sites to ensure the comparison of treatment effects across different climatic regions (Beier et al. 2004; Mikkelsen et al. 2008). Evaluating the impact of climate change treatments on shrubland carbon dynamics was one of the main objectives of this experimental network, and thus a range of methodologies to quantify and trace distinct carbon pools and their fluxes have been applied since 1999. Priority was given to those techniques that minimise disturbances to vegetation and soil to guarantee long-term research.

Here, we review isotope methods that have been applied across this climate change experimental network to study ecosystem carbon dynamics in the plant-soil-air continuum. In particular, we focus on methodologies that: 1) analyse the abundance of naturally rare isotopes of carbon (^{13}C and ^{14}C) and nitrogen (^{15}N) in the different ecosystem compartments to evaluate their responses to climate change; and 2) trace experimentally-induced changes in the isotopic signatures to assess rhizodeposition utilisation by soil biota, by using either $^{13}\text{CO}_2$ pulse labelling, continuous labelling with ^{13}C -depleted CO_2 from Free Air Carbon dioxide Enrichment (FACE), or injections of ^{13}C - and ^{15}N -enriched substrates in the field, and finally 3) manipulate and trace the isotopic composition of C-compounds to analyse C mineralisation by soil microorganisms in laboratory studies. Along-side the methods, data from the field studies are presented as accompanying illustrative boxes, and practical recommendations for the applications of these methodologies at large-scale climate change experiments are outlined in Table 1. The combination of the isotope methods with methods for *in-situ* quantification of

aboveground, root and fungal mycelia biomass will increase our understanding of climate change effects on carbon dynamics with the least possible disturbance.

The experimental climate change network INCREASE

The experimental network for the study of climate change impacts on European shrublands (INCREASE, ‘An Integrated Network on Climate Research Activities on Shrubland Ecosystems’) was established in 1998. The network is comprised of eight shrublands situated across a natural temperature gradient of mean annual temperature from c. 8 °C in the North to c. 16 °C in the South, and a rainfall gradient ranging from 510 mm to 1741 mm from East to West (see Fig. 1 in Reinsch et al. 2017). These sites represent Continental, Atlantic and Mediterranean shrublands. At each site, whole-ecosystem warming and drought treatments were applied in triplicates of 20 m² plots, by using automated retractable curtain constructions (see Beier et al. 2004 and Mikkelsen et al. 2008 for a full description). At one of the Danish sites (DK-BRA), a FACE treatment was installed, and combinations of the climate treatments were established and resulted in a plot size of 9 m² (Mikkelsen et al. 2008). Climatic conditions at the plot level (air temperature, humidity, soil temperature and moisture) were recorded in half-hour or hourly intervals, and main carbon pools and fluxes have been periodically monitored. Most frequent measurements include aboveground plant biomass and composition (Kröel-Dulay et al. 2015), litter production, soil respiration and net ecosystem carbon exchange (Beier et al. 2008; 2009; Lellei-Kovács et al. 2016). Measurements of litter decomposition, soil nitrogen mineralisation (Emmett et al. 2004) and soil microbial biomass and activity (Sowerby et al. 2005) have also been conducted with different periodicity.

Methodologies using natural abundance of carbon isotopes

1 Ecosystem processes reflected by stable isotope fractionation (¹³C and ¹⁵N)

The relative abundance of the rare and heavy stable isotopes of nitrogen (¹⁵N) and carbon (¹³C) compared to the most abundant stable isotope, ¹⁴N and ¹²C respectively, is a signature that reflects the isotopic discrimination associated with gain and loss processes of a given entity. These signatures are expressed as the delta (δ) notation (e.g. δ¹³C and δ¹⁵N in ‰), which is the deviation of the rare isotope abundance in the sample compared to a reference material (Brand

& Coplen 2014; Muccio & Jackson 2009). The naturally occurring background level is termed 'natural abundance' of the given rare stable isotope (Berglund & Wieser 2011). Most natural processes (chemical, physical or enzymatically catalysed) discriminate against heavy isotopes (e.g. ^{13}C , ^{15}N , ^{18}O), which in open systems results in an isotopically depleted product with comparably smaller concentration of the heavy isotopes than its corresponding substrate (Robinson 2001; Fry 2006). If the dominant process rate changes, or if the substrate is exhausted, then the δ value of the product (such as the plant leaf) may significantly change, due to the underlying fractionation. Delta notation is appropriate when dealing with natural samples that are not labelled with excess heavy isotopes (see next sections), and variations at the level of natural abundance are useful for evaluating natural discrimination processes. Importantly, natural abundance cannot be studied if 'overlain' by a study of labelling with heavy isotopes.

Decreases in soil water availability due to drought can alter the isotope signature of both carbon and nitrogen in the aboveground plant biomass. During drought stress, leaves reduce stomatal opening to preserve water. As this happens, the space that confines the air as an immediate source of CO_2 for photosynthesis (the sub-stomatal cavity) becomes a more closed system due to the restriction of the renewal of CO_2 , and as a result a higher proportion of the heavy ^{13}C in CO_2 is fixed by Rubisco (C_3 plants; Tcherkez et al. 2011). Hereby the discrimination against the heavy ^{13}C isotope is decreased. As a consequence, in plants with a C_3 photosynthetic pathway a ^{13}C enrichment in the leaf occurs during drought stress (Cernusak et al. 2013). Indeed, the ^{13}C enrichment at the leaf level is related to an increased intrinsic water use efficiency (WUEi), the ratio of assimilation to stomatal conductance (Farquhar & Richards 1984; Donovan & Ehleringer 1994). Changes in soil water availability may also alter the leaf nitrogen isotope signature by changing the nitrogen availability with soil depth, and thereby the ^{15}N signature of the plant nitrogen source (Lloret et al. 2004). Since $\delta^{15}\text{N}$ is often analysed at the same solid sample as $\delta^{13}\text{C}$ by IRMS (Isotope Ratio Mass Spectrometer), interpretation of $\delta^{15}\text{N}$ can be a useful complement to understand the ecosystem processes. Nitrate and ammonium sources of different origin or at different soil depths can vary in $\delta^{15}\text{N}$ signature (Xue et al. 2009). Hence, if a drought event changes the vertical nitrogen availability in the soil, the plant nitrogen source can shift to a different soil depth possibly causing a change in $\delta^{15}\text{N}$ signature in the leaves. In general, an increase in $\delta^{15}\text{N}$ signature in the leaves indicates a progressive N saturation and/or N losses in the surrounding system because all major pathways of N loss (denitrification, ammonia volatilization and nitrate leaching) cause $\delta^{15}\text{N}$ enrichment of the remaining nitrogen (Peñuelas et al 2000). Interpretation of changes in leaf $\delta^{15}\text{N}$, however,

is not straightforward since leaf $\delta^{15}\text{N}$ signatures might largely depend on mycorrhizal associations and shifts in nitrogen sources between organic and inorganic compounds (the increase in plant $\delta^{15}\text{N}$ values with aridity may also result from increasing reliance on recycled organic N sources as opposed to new inputs) under a drought or warming could influence the leaf $\delta^{15}\text{N}$ as well (Michelsen et al. 1998; Pardo et al. 2006; Andresen et al. 2009).

Across the field sites, the effects of warming and drought on plant ^{13}C and ^{15}N natural abundance was monitored over four years, starting two years after onset of the climate manipulations. Current year shoots or leaves were analysed for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ immediately after each artificially prolonged drought. Plant material was dried at 70°C and ground to a fine powder before analysis for natural abundance values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ using isotope ratio mass spectrometry (IRMS). We expected to find higher $\delta^{13}\text{C}$ values: i) in drought treated plants (compared to control plots) and, ii) in plants growing at drier locations across the precipitation gradient (within a given plant species). Furthermore, we expected iii) the $\delta^{15}\text{N}$ to change in response to drought, as the nitrogen source (depth) is changed (at one location, within-species). Some significant effects of the drought treatment were observed on plant tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Box 1). Differences between years (effect of time) were more pronounced than the effect of the drought treatment for *Populus alba* $\delta^{13}\text{C}$ (HU), *Erica multiflora* $\delta^{15}\text{N}$ (SP) and *Globularium alypum* $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (SP). Only *Calluna vulgaris* showed a significant response to the drought treatment for $\delta^{13}\text{C}$ as hypothesized (Box 1A). For *C. vulgaris*, which was growing at several locations (UK-CL, NL and DK-MOLS), the $\delta^{13}\text{C}$ was higher at drier locations, when compared along the precipitation gradient, and also higher in the drought treatment, at the NL or UK-CL sites (Box 1B). Finally, we found no response of leaf $\delta^{15}\text{N}$ to drought or warming, however, *P. alba* had a much depleted $\delta^{15}\text{N}$ relative to the other species. We attribute these differences to species specific utilization of different nitrogen sources (perhaps more dependent on nitrate at the HU site), or different mycorrhizal associations with higher rates of isotopic fractionation (e.g. ericoid mycorrhiza in *E. multiflora* and *C. vulgaris*; Michelsen et al. 1998; Pardo et al. 2006).

2 Bomb- ^{14}C technique to asses sources of soil respiration

The analysis of natural ^{13}C abundance in CO_2 can be used to distinguish between autotrophic and heterotrophic components of soil respiration in some ecosystems where the sources of respired substrates (i.e. recent photosynthates vs. SOM) have contrasting $\delta^{13}\text{C}$ values. Changes

in $\delta^{13}\text{C}$ values can be observed after vegetation changes, e.g. where C4 plants are introduced in areas previously dominated by C3 vegetation, or vice versa, the so-called C3-C4 shift. In these situations, the $\delta^{13}\text{C}$ signature of plants and soil carbon may differ by up to 10 ‰ (Hanson et al. 2000). The partitioning of soil respiration between historical SOM and recent photosynthates can then be calculated using a linear isotope mixing models (Robinson & Scrimgeour 1995; Hanson et al. 2000). However, such vegetation shifts are rare in natural ecosystems, and therefore the use of ^{13}C natural abundance to differentiate between autotrophic and heterotrophic respiration is limited (but see Millard et al. 2008).

As an alternative to stable carbon isotopes (^{12}C , ^{13}C), the natural radioactive ^{14}C abundance can be used to identify sources of soil respiration. Radiocarbon signatures of recent and older carbon are different as a result of the nuclear bomb tests in the atmosphere during the 1950/60s. These tests led to an increase in the ^{14}C content in the atmospheric CO_2 in the Northern hemisphere, which reached its maximum in 1963 ('bomb peak'). Ever since the subsequent atmospheric nuclear test moratorium, the 'bomb- ^{14}C ' content has decreased due to the dilution with fossil fuel-derived CO_2 in the atmosphere and its incorporation in ocean and terrestrial carbon pools (Trumbore 2009). Through that incorporation, the radiocarbon analysis of ecosystem fluxes provides information about the age of decomposed carbon substrates and can be used to differentiate carbon sources within ecosystems: recently plant-assimilated carbon (autotrophic component of soil respiration) should have a similar radiocarbon signature as the current atmosphere, while the radiocarbon content of older carbon released through SOM mineralisation (heterotrophic component) reflects the year of fixation of that carbon, again a mixing model solution. Several studies have successfully achieved the separation of sources of respiration across ecosystems using the 'bomb- ^{14}C ' method (Gaudinski et al. 2000; Cisneros-Dozal et al. 2006; Czimczik et al. 2006; Schuur & Trumbore 2006; Subke et al. 2011). In these studies, analysis of the ^{14}C - CO_2 signatures of roots and SOM was performed under controlled conditions and collated with analyses of field gas efflux (the mixed pool). Radiocarbon analysis of soil or ecosystem respiration has been used to evaluate the response of a range of ecosystems to different factors of climate change, such as increasing temperatures, decreasing rainfall or permafrost thaw (Borken et al. 2006; Muhr & Borken 2009; Muhr et al. 2009; Schuur et al. 2009).

We tested the effect of experimental warming and drought on the natural abundance of ^{14}C in respired soil CO_2 at early stages of the climate manipulations at the Peaknaze field site (UK-PK). Our hypothesis was that drought increased heterotrophic respiration more than warming

in this seasonally waterlogged soil, due to a greater responsiveness of old soil carbon to drought relative to temperature as a driver (Bol et al. 2003; Domínguez et al. 2015). Therefore, we expected the greatest ^{14}C -enrichment in the field-collected soil respiration samples from the drought plots. Soil efflux samples were collected in the late experimental drought period (September 2011), using a molecular sieve sampling system (Bol et al. 1995; Hardie et al. 2005; Hardie et al. 2009) attached to closed dark respiration chambers placed on the soil overnight. CO_2 was subsequently recovered from the molecular sieve traps for ^{14}C analysis by Accelerator Mass Spectrometry (AMS; Box 2). Soil and root samples were collected to conduct separate incubations to obtain the ^{14}C -signatures of the heterotrophic and autotrophic respiration, respectively. These incubations were performed in leak-tight glass jars with a connection to the molecular sieve sampling system. The results revealed a high heterogeneity of the ^{14}C signature of the soil efflux with no significant effect of the warming treatment, and a trend towards the release of older carbon from the drought plots (although not statistically significant). By comparison with the known record of post-bomb atmospheric ^{14}C - CO_2 concentration (Box 2), the carbon being released from the plots was estimated to have been fixed between six and eight years earlier (M. Dominguez, unpublished).

Methods using *in-situ* ^{13}C labelling to study rhizodeposition utilisation

In-situ pulse labelling with the stable carbon isotope (^{13}C) is an efficient method for evaluating the time lag between carbon assimilation and CO_2 release from soil (Kuzyakov & Gavrichkova 2010). It can be applied to investigate a wide range of processes in the plant-soil-atmosphere continuum (Högberg et al. 2008; Brüggemann et al. 2011; Epron et al. 2012). With this method, a concentrated pulse of ^{13}C -enriched or depleted substrate in the form of CO_2 or of a carbon-containing organic substrate, is released into the undisturbed ecosystem. Subsequently assimilation or heterotrophic consumption will transfer the labelled carbon, and the ^{13}C content of the product and organism will reflect the rate and the quantity of carbon transfer from one pool to another (Studer et al. 2014). Analysis of ^{13}C in specific compounds such as PLFAs is a specific tool to assess the utilisation of different carbon sources by different functional groups of soil biota. The development of ^{13}C labelling has increased the recognition of the central role that rhizodeposition plays in soil carbon cycling (Ostle et al. 2003; Jin & Evans 2010; Kuzyakov & Gavrichkova 2010; Dijkstra et al. 2013).

¹³C-CO₂ pulse labelling

In ¹³C-CO₂ pulse labelling experiments, ¹³C enriched CO₂ is released in closed, intact plant-soil systems during daylight hours, typically for 1.5 to 6 hours, where it is assimilated by the green plant biomass. Plant and soil samples are taken from unlabelled and labelled systems at different time intervals, with a higher sampling frequency within the first 48 hours after the labelling. The allocation of ¹³C to belowground pools (roots, exudates, microbiota) is subsequently analysed, which allows the determination of the fraction of recently fixed carbon actively utilized by e.g. different microbial functional groups. Using ¹³C-CO₂ pulse labelling, several authors demonstrated that the flux of recently photosynthesized carbon to soil microbes occurs very fast, often within a few hours of ¹³CO₂ uptake (Rangel-Castro et al. 2005). The maximum incorporation of ¹³C into microbial RNA occurs within four to eight days after the pulse (Ostle et al. 2003). Fungi typically show the greatest utilisation of plant-derived carbon within the first 48 hours after plant labelling. Lower ¹³C enrichment in bacterial biomarker PLFAs indicates a delay in the utilisation of plant-derived carbon by bacteria, or a greater dependence of bacteria on carbon sources different from recently-fixed carbon. Gram positive bacteria and, in particular actinomycetes, are known to rely less on plant-derived carbon than Gram negative bacteria (Butler et al. 2004; Treonis et al. 2004; Balasooriya et al. 2008; 2013; 2014). The levels of allocation of belowground fixed carbon and the subsequent utilisation by microbes might be affected by a range of factors such as the seasonality of plant activity. Usually, more carbon is allocated belowground towards the end of the growing season (Högberg et al. 2010; Balasooriya et al. 2013), under exposure to elevated atmospheric CO₂ concentrations (Denef et al. 2007; Jin & Evans 2010; Reinsch et al. 2013), under drought conditions (Fuchslueger et al. 2014) or in plants grown on fertile soils (Denef et al. 2009; Paterson et al. 2011).

In the climate change network, several pulse-labelling experiments have been conducted in combination with ¹³C-PLFA analyses to study rhizodeposit utilisation by microbes. At the Clocaenog site (UK-CL) we aimed to study the utilisation of rhizodeposits along a soil moisture gradient, by applying a ¹³C-CO₂ pulse during the late growing-season (August 2011). We used transparent domes of 50 cm diameter and 100 cm height, enclosing individual *C. vulgaris* plants. We applied repeated pulses of ¹³C-CO₂ (99 atom% ¹³C = 99% ¹³C + 1% ¹²C) over eight hours (Box 3). The domes were sealed to a frame which was inserted into the ground at least ten days before the pulse, and had several sealed septa to collect gas samples to estimate the concentration of the ¹³C-labelled CO₂. Plant leaves and soil from the rooting zone were

collected at different times after the labelling, using a higher sampling frequency during the first hours after the pulse. Soils were freeze-dried, sieved to ≤ 5 mm and PLFAs were extracted. Fatty acid methyl esters (FAMES) were analysed by gas chromatography combustion-isotope ratio mass spectrometry (GC-c-IRMS). The main challenge was the low recovery of ^{13}C label in the belowground compartment, especially in individual FAMES. Despite the applied ^{13}C concentration of 99 atom%, the apparent low photosynthetic rates combined with the excessive dilution of the ^{13}C label in the large carbon pools of unlabelled woody branches and root- and microbial biomass resulted in an overall low level of ^{13}C enrichment in the FAMES (Box 3). Similar patterns have also been observed in other pulse labelling experiments (Griffith et al. 2004).

Three pulse-labelling events were conducted at the Brandbjerg site (DK-BRA,) between 2010 and 2013 (Box 3). The Brandbjerg experiment consists of drought and warming manipulations in combination with ambient and elevated levels of CO_2 concentration. The developed experimental setup for pulse-labelling aimed i) to be easily deployable in remote areas, ii) to distribute labelled ^{13}C - CO_2 to as many plots at the same time as possible to ensure similar and constant conditions for CO_2 uptake by the vegetation, and iii) to ensure constant CO_2 concentration available to the vegetation throughout the labelling period. Therefore, a mobile flow-through system suitable for continuous ^{13}C - CO_2 delivery was developed (Box 3): A gas-tight vinyl balloon (~3 m diameter) was filled with CO_2 free synthetic air and mixed with ^{13}C - CO_2 (50 or 99 atom%) that supplied the transparent chambers enclosing the vegetation of interest with air over the duration of the experiments ranging from 4 to 7.5 hours. Air was pumped continuously through gas tight tubing via electric diaphragm pumps (Reinsch & Ambus 2013). The first experiment was conducted at the end of the growing season (October 2010), when we observed the highest allocation of carbon belowground as measured by ^{13}C in soil respiration (Reinsch et al. 2014). The second experiment was conducted in the spring (May 2011) and showed a major allocation of carbon to aboveground structures under elevated atmospheric CO_2 concentration, but carbon allocation to belowground structures was higher in drought plots than in untreated control plots. The allocation of recently-assimilated carbon under warming conditions was similar to that under ambient conditions. The last experiment, conducted in early season 2013 (June), was performed during a period with impeded photosynthetic activity and indicated that labelling performance is poor when vegetation is recovering from harsh winter conditions with bare frost or severe drought conditions (Box 3).

Thus, it is important that the vegetation of interest displays green, photosynthetically active structures to facilitate CO₂ uptake and sufficient labelling of ecosystem carbon pools.

Our studies illustrate the complexity of controlling *in-situ* pulse-labelling experiments in ecosystems dominated by woody plants, which is even more challenging with ¹³C-CO₂ than with ¹⁴C-CO₂ because of their respective atmospheric backgrounds and detection limits (Epron et al. 2012). Ideally, ¹³C doses for *in-situ* use should be carefully tested in trials, considering the nature of the studied vegetation and the compounds to be analysed. If e.g. specific compounds of the soil microbial biomass are the main interest, then strong isotopic doses should be applied, and it is advisable to deploy the ¹³C pulse when plants naturally allocate carbon belowground e.g. when preparing for winter. The ¹³C signal can be increased by using highly labelled ¹³C-CO₂ (99 atom %). However, the usage of a highly enriched CO₂ can potentially lead to blurry signals and has to be applied with caution (Watzinger 2015). Furthermore, ¹³C-CO₂ concentration inside the labelling chamber should be as close as possible to ambient values, because unrealistic high CO₂ concentration will change plant CO₂ uptake. Repeated moderated ¹³C-CO₂ applications during longer exposure times might be more appropriate, but inside closed transparent chambers, temperature and humidity may increase if the labelling period is prolonged, which also affects photosynthetic processes (Epron et al. 2012). Losses of ¹³C due to physical diffusion and adsorption/desorption into the chamber and tubing material should also be considered. In particular, the back-diffusion of the ¹³CO₂ from the soil to the atmosphere which entered the soil pores during the labelling might confound the interpretation of measured belowground respiration (Subke et al. 2009; Selsted et al. 2011). However, when applied properly, the insights into terrestrial carbon allocation will be detailed and novel (Box 3).

2 Free Air Carbon dioxide Enrichment (FACE)-labelling

An alternative method for ¹³C pulse-labelling of vegetation and whole-ecosystems is to utilize the ¹³C-depleted CO₂ in already planned or ongoing FACE experiments. The FACE technique has through decades been used within cropping systems (Kimball 2016), grasslands (Hovenden et al. 2014; Reich et al. 2014; Mueller et al. 2016) and forests (Terrer et al. 2016) experiments, with the primary goal of assessing potential carbon dynamics and enhancement of plant growth (Andresen et al. 2016). As a side effect, the change in carbon isotopic composition of vegetation exposed to FACE-treatment can be used to trace freshly assimilated carbon into soil microbial

biomass, fauna and organic carbon pools. This approach was used at the Brandbjerg site (DK-BRA). The CO₂ used to elevate concentrations of atmospheric CO₂ to 510 ppm had $\delta^{13}\text{C}$ values ranging from -3.0 to -36.7 ‰ throughout 8 years of experimental treatment, with an overall mean of -26.1 ‰. The source of the CO₂ supplied by Air Liquide (Air Liquide, Denmark) was most often a brewery surplus CO₂ as a chemically obtained side product. The FACE mixing of the added CO₂ with ambient CO₂ in the moving air mass resulted in a ^{13}C depletion ranging from -6.7 to -15.6 ‰. On average, this equals a depletion of CO₂ in FACE plots of -4.8 ‰ relative to the atmospheric -8 ‰ average. Ecosystem carbon pools became depleted accordingly, and the FACE ^{13}C depletion acted as a long-term persistent isotope labelling. As a result, soil fauna (Enchytraeids) sampled from each of the climate-treated plots was significantly depleted in $\delta^{13}\text{C}$ by -0.5 to -2.0 ‰ in the CO₂ treatments (Andresen et al. 2011). This was due to translocated ^{13}C substrate through the food web, starting with plant assimilation of ^{13}C -depleted CO₂, followed by plant root exudation and microbial utilization of the ^{13}C depleted substrate and eventual digestion of microbes by enchytraeids. Hereby the freshly supplied carbon source was recognized to be transferred in the natural setting, within a given time scale. Also microbial biomass and PLFAs had different baseline of ^{13}C content in ambient (not-treated) plots compared to CO₂ treated plots (Andresen et al. 2014). This was used for the calculation of ^{13}C enrichments for each PLFA biomarker individually, also illustrating the pathway of newly-assimilated carbon into microbial biomass. A drawback of the ^{13}C -FACE label is again contamination of the surroundings, as even short and small un-planned draft winds can carry the depleted label onto ‘ambient’ plots, and these will most likely be ‘contaminated’ with ^{13}C (though not markedly exposed to high CO₂ concentrations) after some years of FACE activity. Therefore, one needs to collect reference material for the ‘natural abundance’ level well away from the FACE experiment.

3 In situ injection of ^{13}C -enriched substrate solutions

As a much more localized approach to a specific area, *in-situ* addition of ^{13}C - and ^{15}N -enriched substrates directly below the soil surface can be used to assess the competition for the substrate between i) plants and soil microbes, ii) microbial groups, and iii) the effects of the climate change treatments upon the competition for carbon or nitrogen substrates. Much research has focused on the sharing of nitrogen sources between plant and microbes (Kuzyakov & Xu 2013) using *in-situ* soil injections of ^{15}N labelled inorganic nitrogen (ammonium and nitrate) or

organic nitrogen (amino acids) (Bardgett et al., 2003; Sorensen et al., 2008). Once amino acids with dual labelled compounds (^{15}N and ^{13}C) were available for experimental use, double-labelled substrate was used to explore e.g. plant uptake of intact amino acids (Näsholm et al. 2009; Rasmussen et al. 2010), and microbial utilization of carbon substrates (Dungait et al. 2013; Rinnan & Baath 2009).

In a labelling experiment at the DK-BRA site, amino acid injections into the soil were conducted to analyse the impact of the climate treatments on the uptake of free amino acid nitrogen by plants and soil microbes. Dual-labelled glycine ($^{13}\text{C}_2^{15}\text{N}$ -glycine: 99 atom% ^{13}C - of both carbon atoms - and 99 atom% ^{15}N) was added to $20 \times 20 \text{ cm}^2$ sub-plots (Andresen et al. 2009). Each sub-plot received 0.1 L of re-demineralised water labelled with 0.027 g glycine, corresponding to $687 \text{ mg glycine m}^{-2}$ (223 mg C m^{-2} or $0.016 \text{ mg glycine g}^{-1}$ dry weight soil). The label was injected into the soil just below the soil surface with a syringe moved among 16 evenly spaced points of a template, placed on top of the vegetation (Andresen et al. 2009). One day (c. 24 h) after labelling with glycine, soil cores were sampled from the soil surface to 15 cm depth, for determining the relative uptake of the amino acid in plant roots (IRMS solid sample) and soil microbes. As in many other soil labelling experiments, the largest label recovery (measured by ^{15}N recovery since respiratory losses of ^{13}C remain unknown) was found in the total microbial biomass compared to total plant biomass (Kuzyakov & Xu 2013). A subsample of fresh soil was extracted with re-demineralised water, and another set of subsamples was first vacuum-incubated with chloroform for 24 hours to release microbial carbon and nitrogen (Joergensen & Mueller 1996; Brookes et al. 1985), before extraction with re-demineralised water. A third subsample of soil was freeze dried (lyophilized) and later used for PLFA extractions. The ^{13}C enrichment in marker PLFAs thus indicated the activity (vitality) of the specific microbial group (Watzinger 2015). We found that bacteria opportunistically utilised the freshly added glycine substrate, i.e. incorporated ^{13}C , whereas fungi showed only minor or no glycine derived ^{13}C -enrichment (Andresen et al. 2014). In comparison, ^{13}C traced into the microbial community via the ^{13}C - CO_2 pulse label at the same site (DK-BRA) also reached the bacterial community first. Bacteria showed high ^{13}C enrichment compared to fungal groups (Reinsch et al. 2014). This suggests that *in-situ* injection of ^{13}C substrates might be a plausible alternative to mimic rhizodeposition effects. With the direct addition of ^{13}C label to the soil a strong labelling of the microbial community was more easily achieved than with the indirect ^{13}C labelling of microbes via plant assimilated ^{13}C - CO_2 (Box 3).

Use of labelled carbon-compounds to analyse carbon mineralisation by soil microorganisms

Since soil microorganisms have an important role in controlling the availability of nutrients via mineralisation of SOM, our understanding of how microbial functioning in the ecosystem is altered by global change must be improved (Grayston et al. 1997). Microbial catabolic diversity of a soil is directly related to the carbon decomposition function within a soil and potentially provides a sensitive and ecologically relevant measure of the microbial community structure (Garland & Mills 1991). Subsequently, multiple assays have been developed to generate community level physiological profiles (CLPP) that can act as fingerprints of microbial function. Three approaches for measuring CLPP in soils are reported in the literature: (i) Biolog (Garland & Mills 1991); (ii) a substrate-induced respiration (SIR) technique (Degens & Harris 1997); and (iii) MicroResp (Campbell et al. 2003). These methods are all based on quantifying CO₂ respired during the mineralisation of organic carbon compounds that vary in size, charge and structural complexity. The first approach, Biolog MicroPlate™ (Biolog), assesses the catabolic diversity of soil organisms using a microtitre plate by incubating a soil culture in the presence of nutrients and 95 different carbon substrates; respired CO₂ is used to reduce a tetrazolium violet salt, which results in a colour change that can be quantified colorimetrically (Garland & Mills 1991). This approach, however, has been criticized for bias towards fast growing organisms that thrive in culture (Preston-Mafham et al. 2002). In response to the criticism of the Biolog method, Degens & Harris (1997) developed a method based on SIR where individual substrates are added to intact soil and evolved CO₂ is sampled and quantified. Finally, Campbell et al. (2003) combined aspects of both methods (MicroResp™) where the response to carbon substrate addition to soil is measured colorimetrically using a cresol red indicator dye in a microtitre plate format.

Community level physiological profiling of soils samples collected from all treatments across the network was conducted to determine the catabolic utilisation profile, turnover and pool allocation of low molecular weight (LMW) carbon compounds by using a selection of ¹⁴C-labelled substrates. This method enabled the attribution of respired CO₂ to specific metabolic processes that facilitates the quantification and qualification of microbial mineralisation kinetics of substrates varying in structural complexity and recalcitrance. The kinetics of microbial ¹⁴C-CO₂ evolution can be described using a first order exponential decay model (Box 4). The number of terms used in the exponential decay model can be used to explain how microbial kinetics relates time, substrate complexity and carbon pool allocation to, for example,

rapidly cycled labile soil solution carbon, microbial structural carbon and recalcitrant extracellular soil organic carbon (Kuzyakov & Demin 1998; Nguyen & Guckert 2001; Boddy et al. 2007). Attribution of modelled carbon pool sizes and turnover rates to biological function are not only time and substrate dependent. Therefore, soil physical, biological and chemical interactions may be miss attributed to biological function. Indeed, the lack of knowledge and techniques available to examine the interaction between discrete carbon pools (Glanville et al, 2016). Using the half-life of ^{14}C labelled carbon in soil solution we were able to examine the environmental gradient of the warming treatment across the climate change network and identified that temperature becomes rate limiting for microbial uptake of carbon from the soil solution pool at $< 10.5\text{ }^{\circ}\text{C}$. We also showed that experimentally manipulated warming simply speeds up the catabolic utilisation of labile LMW carbon in a predictable pattern (Box 4).

Conclusions and recommendations

Stable isotope studies provide insightful information about carbon (and nitrogen) fluxes through the plant-soil-atmosphere continuum with minimal disturbance to the system, and contribute to advance our understanding of climate change impacts on aboveground-belowground linkages. However, their application is not exempt from difficulties and disadvantages. To keep a high caution and avoid mistakes, our collective recommendations for applying the described methods are provided and addressed in Table 1.

In-situ pulse-labelling studies are powerful to analyse short-term carbon fluxes in the plant-soil system, but there are major seasonality constraints to the distribution of the label throughout the ecosystem compartments, *i.e.* the seasonality of carbon allocation belowground due to changing plant activity, or the plant health status which determines the amount of tracer entering the system. A significant challenge was the achievement of sufficient ^{13}C enrichment in microbial biomass in *in-situ* ^{13}C - CO_2 pulse-labelling studies, where the pools of background carbon in the studied compartments were high and hence diluted the ^{13}C signal. This was less of a problem for ^{14}C studies, due to more sensitive analysis methods.

Importantly, field plots previously ‘contaminated’ by highly enriched isotope labelling should be considered potentially inoperable for further scientific isotope studies using the natural abundance approach. However, plant and soil structures remain largely undisturbed. In outlook for setting up a large-scale climate manipulation, areas that have not been previously used for experimental work with isotopes should consequently be selected. In particular, the ‘bomb-C’

method is very sensitive to the contamination of soil or plant samples with ^{14}C -enriched material, and thus its application should be limited to sites and facilities where no ^{14}C -labelling work has been conducted. Additionally, it should be noted that any history of fertilization might also alter the natural isotope abundance of ecosystem compartments (in particular ^{15}N signatures), potentially confounding experimental results. Furthermore, military training grounds, public recreational activity and vicinity of traffic are known to potentially contaminate the soil with ‘artificial compounds’ which might interfere with delicate measurements on HPLC and GC-MS systems.

Incubation studies with isotope labelled carbon compounds *in-situ* or *in-vitro* are relatively fast to conduct and produce insightful data. The rapid utilisation of labelled substrates by soil microorganisms, occurring immediately upon application, is a controlling factor for the timing of the experimental work both at field and lab conditions. Hence, a sampling scheme needs to be carefully planned before experiment initiation. Furthermore, pre-obtained knowledge of site specific plant and microbial activity, substrate affinity and natural substrate concentrations is important for planning any tracer application experiment (Table 1).

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References

Andresen, L.C., Michelsen, A., Jonasson, S., Beier, C. & Ambus, P. (2009) Glycine uptake in heath plants and soil microbes s responds to elevated temperature, CO_2 and drought. *Acta OEcologica*, 313, 283-295.

586 Andresen, L.C., Konestabo, H.S., Maraldo, K., Holmstrup, M., Ambus, P., Beier, C. &
 587 Michelsen, A. (2011) Organic matter flow in the food web at a temperate heath under
 588 multifactorial climate change. *Rapid Communications in Mass Spectrometry*, 25, 1485-1496.

589 Andresen, L.C., Dungait, J.A.J., Bol, R., Selsted, M.B., Ambus, P. & Michelsen, A. (2014)
 590 Bacteria and Fungi Respond Differently to Multifactorial Climate Change in a Temperate
 591 Heathland, Traced with ¹³C-Glycine and FACE CO₂. *PlosOne*, 9, e85070.

592 Balasooriya, W.K., Denef, K., Peters, J., Verhoest, N.E.C. & Boeckx, P. (2008) Vegetation
 593 composition and soil microbial community structural changes along a wetland hydrological
 594 gradient. *Hydrology and Earth System Sciences*, 12, 277-291.

595 Balasooriya, W., Huygens, D., Denef, K., Roobroeck, D., Verhoest, N.C. & Boeckx, P. (2013)
 596 Temporal variation of rhizodeposit-C assimilating microbial communities in a natural wetland.
 597 *Biology and Fertility of Soils*, 49, 333-341.

598 Balasooriya, W., Denef, K., Huygens, D. & Boeckx, P. (2014) Translocation and turnover of
 599 rhizodeposit carbon within soil microbial communities of an extensive grassland ecosystem.
 600 *Plant and Soil*, 376, 61-73

601 Beier, C., Emmett, B.A., Gundersen, P., Tietema, A., Peñuelas, J., Estiarte, M., Gordon, C.
 602 Gorissen, A., Llorens, L., Roda, F. & Williams, D. (2004) Novel approaches to study climate
 603 change effects on terrestrial ecosystems in the field: drought and passive night-time warming.
 604 *Ecosystems*, 7, 583-597.

605 Beier, C., Emmett, B.A., Peñuelas, J., Schmidt, I.K., Tietema, A., Estiarte, M., Gundersen, P.,
 606 Llorens, L., Riis-Nielsen, T., Sowerby, A. & Gorissen, A. (2008) Carbon and nitrogen cycles
 607 in European ecosystems respond differently to global warming. *Science of the Total*
 608 *Environment*, 407, 692-697.

609 Beier, C., Emmett, B.A., Tietema, A., Schmidt, I.K., Peñuelas, J., Kovács-Lang, E., Duce, P.,
 610 De Angelis, P. Gorissen, A., Estiarte, M., de Dato, G.D., Sowerby, A., Kröel-Dulay, G., Lellei-
 611 Kovács, E., Kull, O., Mänd, P., Petersen, H., Gjelstrup, P. & Spano, D. (2009) Carbon and
 612 nitrogen balances for six shrublands across Europe. *Global Biogeochemical Cycles*, 23, Art.
 613 No. GB4008.

614 Boddy, E., Hill, P.W., Farrar, J.F. & Jones, D.L. (2007) Fast turnover of low molecular weight
 615 components of the dissolved organic carbon pool of temperate grassland field soils. *Soil Biology*
 616 *and Biochemistry*, 39, 827-835.

617 Bol, R. & Harkness, D.D. (1995) The use of zeolite molecular sieves for trapping low
618 concentrations of CO₂ from environmental atmospheres. *Radiocarbon*, 37, 643-647

619 Bol, R., Bolger, T., Cully, R. & Little, D. (2003) Recalcitrant soil organic material mineralise
620 more efficiently at higher temperatures. *Journal of Plant Nutrition and Soil Science*, 166, 300-
621 307.

622 Borken, W., Savage, K., Davidson, E.A. & Trumbore, S.E. (2006) Effects of experimental
623 drought on soil respiration and radiocarbon efflux from a temperate forest soil. *Global Change*
624 *Biology*, 12, 177-193.

625 Brand, W.A. & Coplen, T.B. (2014) Stable isotope deltas: tiny, yet robust signatures in nature.
626 *Isotopes in Environmental and Health Studies*, 48, 393-409;

627 Brookes, P.C., Landman, A., Pruden, G., & Jenkinson, D.S. (1985) Chloroform fumigation and
628 the release of soil nitrogen: A rapid direct extraction method to measure microbial biomass
629 nitrogen in soil. *Soil Biology and Biochemistry*, 17, 837-842.

630 Brüggemann, N., Gessler, A., Kayler, Z., Keel, S.G., Badeck, F., Barthel, M., Boeckx, P.,
631 Buchmann, N., Brugnoli, E., Esperschuetz, J., Gavrichkova, O., Ghashghaie, J., Gomez-
632 Casanovas, N., Keitel, C., Knohl, A., Kuptz, D., Palacio, S., Salmon, Y., Uchida, Y. & Bahn,
633 M. (2011) Carbon allocation and carbon isotope fluxes in the plant-soil-atmosphere continuum:
634 a review. *Biogeosciences*, 8, 3457-3489.

635 Butler, J.L., Bottomley, P.J., Griffith, S.M. & Myrold, D.D. (2004) Distribution and turnover
636 of recently fixed photosynthate in ryegrass rhizospheres. *Soil Biology and Biochemistry*, 36,
637 371-382.

638 Campbell, C.D., Chapman, S.J., Cameron, C.D., Davidson, M.S. & Potts, J.M., (2003) A rapid
639 microtitre plate method to measure carbon dioxide evolved from carbon substrate amendments
640 so as to determine the physiological profiles of soil microbial communities by using whole soil.
641 *Applied and Environmental Microbiology*, 69, 3593-3599.

642 Cernusak, L.A., Ubierna, N., Winter, K., Holtum, J.A.M., Marshall, J.D. & Farquhar, G.D.
643 (2013) Environmental and physiological determinants of carbon isotope discrimination in
644 terrestrial plants. *New Phytologist*, 200, 950–965.

645 Chapin, F.S., McFarland, J., David McGuire, A., Euskirchen, E. S., Ruess, R. W., & Kielland,
646 K. (2009) The changing global carbon cycle: linking plant-soil carbon dynamics to global
647 consequences. *Journal of Ecology*, 97, 840–850.

648 Cisneros-Dozal, L.M., Trumbore, S. & Hanson, P.J. (2006) Partitioning sources of soil-respired
649 CO₂ and their seasonal variation using a unique radiocarbon tracer. *Global Change Biology*,
650 12, 194-204.

651 Czimczik, C.I., Trumbore, S.E., Carbone, M.S. & Winston, G.C. (2006) Changing sources of
652 soil respiration with time since fire in a boreal forest. *Global Change Biology*, 12, 957-971.

653 de Graaff, M.A., Six, J., & van Kessel, C. (2007) Elevated CO₂ increases nitrogen
654 rhizodeposition and microbial immobilization of root-derived nitrogen. *New Phytologist*, 173,
655 778-786.

656 Degens, B.P. & Harris, J.A. (1997) Development of a physiological approach to measuring the
657 catabolic diversity of soil microbial communities. *Soil Biology and Biochemistry*, 29: 1309-
658 1320.

659 Denman, K.L., Brasseur, G., Chidthaisong, A., Ciais, P., Cox, P.M., Dickinson, R.E.,
660 Hauglustaine, D., Heinze, C., Holland, E., Jacob, D., Lohmann, U., Ramachandran, S., da Silva
661 Dias, P.L., Wofsy, S.C., & Zhang, X. (2007) Couplings between changes in the climate system
662 and biogeochemistry. In: Solomon, S., Qin, D., Manning, M., Chen, M.Z., Marquis, M., Averyt,
663 K.B., Tignor, M. & Miller, H.L. (eds.) *Climate change 2007: the physical science basis*.
664 Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental
665 Panel on Climate Change. Cambridge University Press, Cambridge, pp 500-587.

666 Denef, K., Bubenheim, H., Lenhart, K., Vermeulen, J., Van Cleemput, O., Boeckx, P. & Müller,
667 C. (2007) Community shifts and carbon translocation within metabolically-active rhizosphere
668 microorganisms in grasslands under elevated CO₂. *Biogeosciences*, 4, 769-779.

669 Denef, K., Roobroeck, D., Manimel Wadu, M.C.W., Lootens, P. & Boeckx, P. (2009) Microbial
670 community composition and rhizodeposit-carbon assimilation in differently managed
671 temperate grassland soils. *Soil Biology and Biochemistry*, 41, 144-153.

672 Dijkstra, F.A., Carrillo, Y., Pendall, E. & Morgan, J.A. (2013) Rhizosphere priming: a nutrient
673 perspective. *Frontiers in Microbiology*, 4, 216.

674 Domínguez, M.T, Sowerby, A., Smith, A., Robinson, D., Van Baarsel, S., Mills, R.E., Marshall,
675 M., Koller, E., Lebron, I., Hall, J., & Emmett, B. (2015) Sustained impact of drought on wet
676 shrublands mediated by soil physical changes. *Biogeochemistry*, 122, 151-163.

677 Donovan, L.A. & Ehleringer, J.R. (1994) Carbon isotope discrimination, water-use efficiency,
678 growth, and mortality in a natural shrub population. *Oecologia*, 100, 347-354.

679 Dungait, J.A.J., Kemmitt, S.J., Michallon, L., Guo, S., Wen, Q., Brookes, P.C. & Evershed,
680 R.P. (2013) The variable response of soil microorganisms to trace concentrations of low
681 molecular weight organic substrates of increasing complexity. *Soil Biology & Biochemistry*, 64,
682 57–64.

683 Emmett, B.A., Beier, C., Estiarte, M., Tietema, A., Kristensen, H.L., Williams, D., Peñuelas,
684 J., Schmidt, I. & Sowerby, A. (2004) The response of soil processes to climate change: results
685 from manipulation studies of shrublands across and environmental gradient. *Ecosystems*, 7,
686 625-637.

687 Epron, D., Bahn, M., Derrien, D., Lattanzi, F.A., Pumpanen, J., Gessler, A., Hörgberg, P.,
688 Maillard, P., Dannoura, M., Gérant, D. & Buchmann, N. (2012) Pulse-labelling trees to study
689 carbon allocation dynamics: a review of methods, current knowledge and future prospects. *Tree*
690 *Physiology*, 32, 776-798.

691 Farquhar, G.D. & Richards, R.A. (1984) Isotopic composition of plant carbon correlates with
692 water-use efficiency of wheat genotypes. *Australian Journal of Plant Physiology*, 11, 539-552.

693 Friedlingstein, P., Cox, P., Betts, R., Bopp, L., von Bloh, W., Brovkin, V., Cadule, P., Doney,
694 S., Eby, M., Fung, I., Bala, G., John, J., Jones, C., Joos, F., Kato, T., Kawamiya, M., Knorr, W.,
695 Lindsay, K., Matthews, H.D., Raddatz, T., Rayner, P., Reick, C., Roeckner, E., Schnitzler, K.G.,
696 Schnur, R., Strassmann, K., Weaver, A.J., Yoshikawa, C., & Zeng, N. (2006) Climate–carbon
697 cycle feedback analysis: results from the C4MIP model intercomparison. *Journal of Climate*,
698 19, 3337-3353

699 Fry. B. (2006) *Stable Isotope Ecology*. Springer, New York.

700 Fuchslueger, L., Bahn, M., Fritz, K., Hasibeder, R. & Richter, A. (2014) Experimental drought
701 reduces the transfer of recently fixed plant carbon to soil microbes and alters the bacterial
702 community composition in a mountain meadow. *New Phytologist*, 201, 916-927.

703 Gaudinski, J.B., Trumbore, S.E., Davidson, E.A. & Shuhui, Z. (2000) Soil carbon cycling in a
704 temperate forest: radiocarbon-based estimates of residence times, sequestration rates and
705 partitioning of fluxes. *Biogeochemistry*, 51, 33-69.

706 Garland, J.L. & Mills, A.L. (1991) Classification and characterisation of heterotrophic
707 microbial communities on the basis of patterns of community-level sole-carbon-source
708 utilisation. *Applied and Environmental Microbiology*, 57, 2351-2359.

709 Glanville, H.C., Hill, P.W., Schnepf, A., Oburger, E., Jones, D.L. (2016) Combined use of
 710 empirical data and mathematical modelling to better estimate the microbial turnover of
 711 isotopically labelled carbon substrates in soil. *Soil Biology and Biogeochemistry*, 94, 154-168.

712 Grayston, S.J., Vaughan, D. & Jones, D. (1997) Rhizosphere carbon flow in trees, in
 713 comparison with annual plants: the importance of root exudation and its impact on microbial
 714 activity and nutrient availability. *Applied Soil Ecology*, 5, 29-56.

715 Gunina, A. & Kuzyakov, Y. (2014) Pathways of litter C by formation of aggregates and SOM
 716 density fractions: Implications from ^{13}C natural abundance. *Soil Biology and Biogeochemistry*,
 717 71, 95-104.

718 Hanson, P.J., Edwards, N.T., Garten, C.T. & Andrews, J.A. (2000) Separating root and soil
 719 microbial contributions to soil respiration: a review of methods and observations.
 720 *Biogeochemistry*, 48, 115-146.

721 Hardie, S.M.L., Garnett, M.H., Fallick, A.E., Rowland, A.P. & Ostle, N.J. (2005) Carbon
 722 dioxide capture using a zeolite molecular sieve sampling system for isotopic studies ^{13}C and
 723 ^{14}C of respiration. *Radiocarbon*, 47, 441-451.

724 Hardie, S.M.L., Garnett, M.H., Fallick, A.E., Ostle, N.J. & Rowland, A.P. (2009) Bomb- ^{14}C
 725 analysis of ecosystem respiration reveals that peatland vegetation facilitates release of old
 726 carbon. *Geoderma*, 153, 393-401.

727 Hovenden, M.J., Newton P.C.D. & Wills, K.E. Seasonal not annual rainfall determines
 728 grassland biomass response to carbon dioxide. *Nature*, 511, 583-586.

729 Högberg, P., Högberg, M.N., Göttlicher, S.G., Betson, N.R., Keel, S.G., Metcalfe, D.B.,
 730 Campbell, C., Schindelbacher, A., Hurry, V., Lundmark, T., Linder, S. & Näsholm, T. (2008)
 731 High temporal resolution tracing of photosynthate carbon from the tree canopy to forest soil
 732 microorganisms. *New Phytologist*, 177, 220-228.

733 Högberg, M.N. & Högberg, P. (2002) Extramatrical ectomycorrhizal mycelium contributes
 734 one-third of microbial biomass and produces, together with associated roots, half the dissolved
 735 organic carbon in a forest soil. *New Phytologist*, 154, 791-795.

736 Högberg, M.N., Briones, M.J.I., Keel, S.G., Metcalfe, D.B., Campbell, C., Midwood, A.J.,
 737 Thornton, B., Hurry, V., Linder, S., Näsholm, T. & Högberg, P. (2010) Quantification of effects
 738 of season and nitrogen supply on tree below-ground carbon transfer to ectomycorrhizal fungi
 739 and other soil organisms in a boreal pine forest. *New Phytologist*, 187, 485-493.

740 IPCC Intergovernmental Panel on Climate Change (2013) Working group I contribution to the
 741 IPCC fifth assessment report (AR5), Climate Change 2013: The Physical Science Basis.

742 Jin, V.L. & Evans, R.D.E. (2010) Microbial ^{13}C utilisation patterns via stable isotope probing
 743 of phospholipid biomarkers in Mojave Desert soils exposed to ambient and elevated
 744 atmospheric CO_2 . *Global Change Biology*, 16, 2334-2344.

745 Joergensen, R.G. & Mueller, T. (1996) The fumigation-extraction method to estimate soil
 746 microbial biomass: calibration of the ken value. *Soil Biology and Biochemistry*, 28, 33–37.

747 Kimball, B.A. (2016) Crop responses to elevated CO_2 and interactions with H_2O , N, and
 748 temperature. *Current Opinion in Plant Biology*, 31, 36-43.

749 Kuzyakov, Y. & Demin, V. (1998) CO_2 efflux by rapid decomposition of low
 750 molecular organic substances in soils. *Sciences of Soils*, 3, 11–22.

751 Kuzyakov, Y. & Gavrichkova, O. (2010) Time lag between photosynthesis and carbon dioxide
 752 efflux from soil: a review of mechanisms and controls. *Global Change Biology*, 16, 3386-3406.

753 Kuzyakov, Y. & Xu, X. (2013) Competition between roots and microorganisms for nitrogen:
 754 mechanisms and ecological relevance. *New Phytologist*, 198, 656–669.

755 Kröel-Dulay, G., Ransijn, J., Schmidt, I.K., Beier, C., De Angelis, P., de Dato, G., Dukes, J.S.,
 756 Emmett, B., Estiarte, M., Garadnai, J., Kongstad, J., Kovacs-Lang, E., Larsen, K.S., Liberati,
 757 D., Ogaya, R., Riis-Nielsen, T., Smith, A.R., Sowerby, A., Tietema, A. & Peñuelas, J. (2015)
 758 Increased sensitivity to climate change in disturbed ecosystems. *Nature communications* 6,
 759 6682.

760 Leakey, A.D.B., Ainsworth, E.A., Bernacchi, C.J., Rogers, A., Long, S.P. & Ort, D.R. (2009)
 761 Elevated CO_2 effects on plant carbon, nitrogen and water relations: six important lessons from
 762 FACE. *Journal of Experimental Botany*, 60, 2859-2876.

763 Lellei-Kovács, E., Botta-Dukat, Z., de Dato, G., Estiarte, M., Guidolotti, G., Kopittke, G.R.,
 764 Kovacs-Lang, E., Kroel-Dulay, G., Larsen, K.S., Penuelas, J., Smith, A.R., Sowerby, A.,
 765 Tietema, A. & Schmidt I.K. (2016) Temperature dependence of soil respiration modulated by
 766 thresholds in soil water availability across European shrubland ecosystems. *Ecosystems*, 19,
 767 1460–1477.

768 Lichter, J., Barron, S.H., Bevacqua, C.E., Finzi, A.C. Irving, K.E., Stemmler, E.A. &
 769 Schlesinger, W.H. (2005) Soil carbon sequestration and turnover in a pine forest after six years
 770 of atmospheric CO_2 enrichment. *Ecology*, 86, 1835:1847.

771 Lloret, F., Siscart, D., & Dalmases, C. (2004). Canopy recovery after
 772 drought dieback in holm-oak Mediterranean forests of Catalonia (NE
 773 Spain). *Global Change Biology*, 10, 2092–2099.

774 Michelsen, A., Quarmby, C., Sleep, D et al.. (1998) Vascular plant N-15 natural abundance in
 775 heath and forest tundra ecosystems is closely correlated with presence and type of mycorrhizal
 776 fungi in roots. *Oecologia*, 115, 406-418.

777 Millard, P., Midwood, A.J., Hunt, J.E., Whitehead, D. & Boutton, T.W. (2008) Partitioning soil
 778 surface CO₂ efflux into autotrophic and heterotrophic components, using natural gradients in
 779 soil $\delta^{13}\text{C}$ in an undisturbed savannah soil. *Soil Biology and Biochemistry*, 40, 1575–1582.

780 Mikkelsen, T.N., Beier, C., Jonasson, S., Holmstrup, M., Schmidt, I.K., Ambus, P., Pilegaard,
 781 K., Michelsen, A., Albert, K., Andresen, L.C., Arndal, M.F., Bruun, N., Christensen, S.,
 782 Danbæk, S., Gundersen, P., Jørgensen, P., Linden, L.G., Kongstad, J., Maraldo, K., Priemé, A.,
 783 Riis-Nielsen, T., Ro-Poulsen, H., Stevnbak, K., Selsted, M.B., Sørensen, P., Larsen, K.S.,
 784 Carter, M.S., Ibrom, A., Martinussen, T., Miglietta, F. & Sverdrup, H. (2008) Experimental
 785 design of multifactor climate change experiments with elevated CO₂, warming and drought:
 786 the CLIMAITE project. *Functional Ecology*, 22, 185-195.

787 Muccio, Z. & Jackson, G.P. (2009) Isotope ratio mass spectrometry. *Analyst*, 134, 213–222.

788 Mueller, K.E., Blumenthal, D.M., Pendall, E., Carrillo, Y., Dijkstra, F.A., Williams, D.G.,
 789 Follett, R.F. & Morgan, J.A. (2016). Impacts of warming and elevated CO₂ on a semi-arid
 790 grassland are non-additive, shift with precipitation, and reverse over time. *Ecology Letters*, 19,
 791 956–966.

792 Muhr, J.A.N., Borken, W. & Matzner, E. (2009) Effects of soil frost on soil respiration and its
 793 radiocarbon signature in a Norway spruce forest soil. *Global Change Biology*, 15, 782-793.

794 Muhr, J. & Borken, W. (2009) Delayed recovery of soil respiration after wetting of dry soil
 795 further reduces C losses from a Norway spruce forest soil. *Journal of Geophysical Research-*
 796 *Biogeosciences*, 114, G04023.

797 Nguyen, C. & Guckert, A. (2001) Short-term utilisation of ¹⁴C-[U]glucose by soil
 798 microorganisms in relation to carbon availability. *Soil Biology and Biochemistry*, 33, 53-60.

799 Ostle, N., Whiteley, A.S., Bailey, M.J., Sleep, D., Ineson, P. & Manefield, M. (2003) Active
 800 microbial RNA turnover in a grassland soil estimated using a ¹³CO₂ spike. *Soil Biology and*
 801 *Biochemistry*, 35, 877-885.

802 Näsholm, T., Kielland, K. & Ganeteg, U. (2009) Uptake of Organic Nitrogen by Plants *The*
803 *New Phytologist*, 182, 31-48.

804 Paterson, E., Neilson, R., Midwood, A.J., Osborne, S.M., Sim, A., Thornton, B. & Millard, P.
805 (2011) Altered food web structure and C-flux pathways associated with mineralisation of
806 organic amendments to agricultural soil. *Applied Soil Ecology*, 48, 107-116.

807 Pardo, L.H., Templer, P.H., Goodale, C.L., Duke, S., Groffman, P.M., Adams, M.B. Boeckx,
808 P., Goggs, J., Campbell, J., Colman, B., Compton, J., Emmett, B., Gundersen, P., Kjonaas, J.,
809 Lovett, G., Mack, M., Magill, A., Mbila, M., Mitchell, M.J., McGee, G., McNulty, S.,
810 Nadelhoffer, K., Ollinger, S., Ross, D., Rueth, H., Rustad, L., Schaberg, P., Schiff, S., Schlepp,
811 P., Phillips, DA, Fox, TC & Six J (2006) Root exudation (net efflux of amino acids) may
812 increase rhizodeposition under elevated CO₂. *Global Change Biology*, 12, 561-567.

813 Phillips, D. A., Fox, T. C., & SIX, J. (2006) Root exudation (net efflux of amino acids) may
814 increase rhizodeposition under elevated CO₂. *Global Change Biology*, 12, 561–567.

815 Preston-Mafham, J., Boddy, L. & Randerson, P.F., (2002) Analysis of microbial community
816 functional diversity using sole-carbon source utilisation profiles—a critique. *FEMS*
817 *Microbiology Ecology*, 42, 1–14.

818 Rangel-Castro, J.I., Killham, K., Ostle, N., Nicol, G.W., Anderson, I.C., Scrimgeour, C.M.,
819 Ineson, P., Meharg, A. & Prosser, J.I. (2005) Stable isotope probing analysis of the influence
820 of liming on root exudate utilisation by soil microorganisms. *Environmental Microbiology*, 7,
821 828-838.

822 Rasmussen, J., Sauheitl, L., Eriksen, J. & Kuzyakov, Y. (2010) Plant uptake of dual-labeled
823 organic N biased by inorganic C uptake: Results of a triple labeling study. *Soil Biology &*
824 *Biochemistry*, 42, 524e527

825 Reich, P.B., Hobbie, S.E. & Lee, T.D. (2014) Plant growth enhancement by elevated CO₂
826 eliminated by joint water and nitrogen limitation. *Nature Geoscience*, 7, 920–924.

827 Reinsch, S., Arndal, M.F., Schmidt, I.K., Domínguez, M.T., Beier C., de Dato, G., Devan S.,
828 Emmett, B.A., Estiarte, M., Godbold, D.L., Sandén, H., Kröel-Dulay, G., Larsen, K.S., Lellei-
829 Kovács, E., Mason, S., Peñuelas, J., Riis-Nielsen, T., Tietema, A., and Andresen, L.C. Methods
830 for non-destructive assessment of terrestrial carbon pools and fluxes in shrublands in long-term
831 climate change experiments. *Submitted companion paper to Methods in Ecology and Evolution*.

832 Reinsch, S., Michelsen, A., Sárossy, Z., Egsgaard, H., Schmidt, I.K., Jakobsen, I. & Ambus, P.
833 (2014) Short-term utilisation of carbon by the soil microbial community under future climatic
834 conditions in a temperate heathland. *Soil Biology and Biochemistry*, 68, 9-19.

835 Reinsch, S. & Ambus, P. (2013) In situ $^{13}\text{CO}_2$ pulse-labeling in a temperate heathland
836 development of a mobile multi-plot field setup. *Rapid Communications in Mass Spectrometry*,
837 27, 1417-1428.

838 Rinnan, R. & Baaath, E. (2009) Differential utilization of carbon substrates by bacteria and
839 fungi in tundra soil. *Applied and Environmental Microbiology*, 75, 3611–3620.

840 Robinson, D. (2001) $\delta^{15}\text{N}$ as an integrator of the nitrogen cycle. *TRENDS in Ecology and*
841 *Evolution*, 16, 153-162.

842 Robinson, D. & Scrimgeour, C.M. (1995) The contribution of plant C to soil CO_2 measured
843 using ^{13}C . *Soil Biology and Biochemistry*, 27, 1653–1656.

844 Ruess, L., & Chamberlain, P. M. (2010). The fat that matters: Soil food web analysis using fatty
845 acids and their carbon stable isotope signature. *Soil Biology and Biochemistry*, 42, 1898–1910.

846 Schuur, E.A. & Trumbore, S.E. (2006) Partitioning sources of soil respiration in boreal black
847 spruce forest using radiocarbon. *Global Change Biology*, 12, 165-176.

848 Schuur, E.A.G., Vogel, J.G., Crummer, K.G., Lee, H., Sickman, J.O. & Osterkamp, T.E. (2009)
849 The effect of permafrost thaw on old carbon release and net carbon exchange from tundra.
850 *Nature*, 459, 556-559.

851 Schrumpf, M., Kaiser, K., Guggenberger, G., Persson, T., Kogel-Knabner, I. & Schulze, E.D.
852 (2013) Storage and stability of organic carbon in soils as related to depth, occlusion within
853 aggregates, and attachment to minerals. *Biogeosciences*, 10, 1675-1691.

854 Selsted, M. B., Ambus, P., Michelsen, A., van der Linden, L., Larsen, K. S., Pilegaard, K.,
855 Mikkelsen, T. N. & Beier, C. (2011) Measurement of carbon dioxide fluxes in a free-air carbon
856 dioxide enrichment experiment using the closed flux chamber technique. *Atmospheric*
857 *Environment*, 45, 208-214.

858 Sorensen, P.L., Michelsen, A., & Jonasson, S. (2008) Nitrogen uptake during one year in
859 subarctic plant functional groups and in microbes after long-term warming and fertilization.
860 *Ecosystems*, 11, 1223–1233.

861 Sowerby, A., Emmett, B., Beier, C., Tietema, A., Peñuelas, J., Estiarte, M., Van Meeteren,
862 M.J.M., Hughes, S. & Freeman, C. (2005) Microbial community changes in heathland soil

863 communities along a geographical gradient: interaction with climate change manipulations. *Soil*
864 *Biology and Biochemistry*, 37: 1805-1813.

865 Sowerby, A., B.A. Emmett, A. Tietema & C. Beier (2008) Contrasting effects of repeated
866 summer drought on soil carbon efflux in hydric and mesic heathland soils. *Global Change*
867 *Biology*, 14, 2388-2404.

868 Studer, M.S., Siegwolf, R.T.W. & Abiven, S. (2014) Carbon transfer, partitioning and residence
869 time in the plant-soil system: a comparison of two $^{13}\text{CO}_2$ labelling techniques. *Biogeosciences*,
870 11, 1637-1648.

871 Subke, J.A., Vallack, H.M., Tord, M., Keel, S.G., Metcalfe, D.B., Högberg, P. & Ineson, P.
872 (2009) Short-term dynamics of abiotic and biotic soil $^{13}\text{CO}_2$ effluxes after in situ $^{13}\text{CO}_2$ pulse-
873 labelling of a boreal pine forest. *New Phytologist*, 183, 349–357.

874 Subke, J.A., Voke, N.R., Leronni, V., Garnett, M.H. & Ineson, P. (2011) Dynamics and
875 pathways of autotrophic and heterotrophic soil CO_2 efflux revealed by forest girdling. *Journal*
876 *of Ecology*, 99, 186-193.

877 Tcherkez, G., Mahe, A., & Hodges M. (2011) $^{12}\text{C}/^{13}\text{C}$ fractionations in plant primary
878 metabolism. *Trends in Plant Science*, 16, 499-506.

879 Terrer, C., Vicca, S., Hungate, B.A., Phillips, R.P. & Prentice, I.C., 2016. Mycorrhizal
880 association as a primary control of the CO_2 fertilization effect. *Science*, 353, 72–74.

881 Treonis, A.M., Ostle, N.J., Stott, A.W., Primrose, R., Grayston, S.J. & Ineson, P. (2004)
882 Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope
883 probing of PLFAs. *Soil Biology and Biochemistry*, 36, 533-537

884 Trumbore, S.E. (2009) Radiocarbon and Soil Carbon Dynamics. *Annual Reviews on Earth*
885 *Planetary Sciences*, 37, 47–66.

886 Verdú, M. (2000) Ecological and Evolutionary Differences between Mediterranean Seeders and
887 Resprouters. *Journal of Vegetation Science*, 11, 265–268.

888 Watzinger, A. (2015) Microbial phospholipid biomarkers and stable isotope methods help
889 reveal soil functions. *Soil Biology and Biochemistry*, 86, 98-107.

890 Wessel, W.W., Tietema, A., Beier, C., Emmett, B.A., Peñuelas, J., & Riis-Nielsen, T. (2004)
891 A Qualitative Ecosystem Assessment for Different Shrublands in Western Europe under Impact
892 of Climate Change. *Ecosystems*, 7, 662–671.

893 Xue, D., Botte, J., De Baets, B., Accoe, F., Nestler, A., Taylor, P., Van Cleemput, O., Berglund,
894 M. & Boeckx, P. (2009) Present limitations and future prospects of stable isotope methods for
895 nitrate source identification in surface- and groundwater. *Water research*, 43, 1159-1170.

896 Zak, D.R., Pregitzer, K.S., King, J.S. & Holmes, W.E. (2000) Elevated atmospheric CO₂, fine
897 roots and the response of soil micro-organisms: a review and hypothesis. *New Phytologist*, 147,
898 201-222.

899

900 **Table**

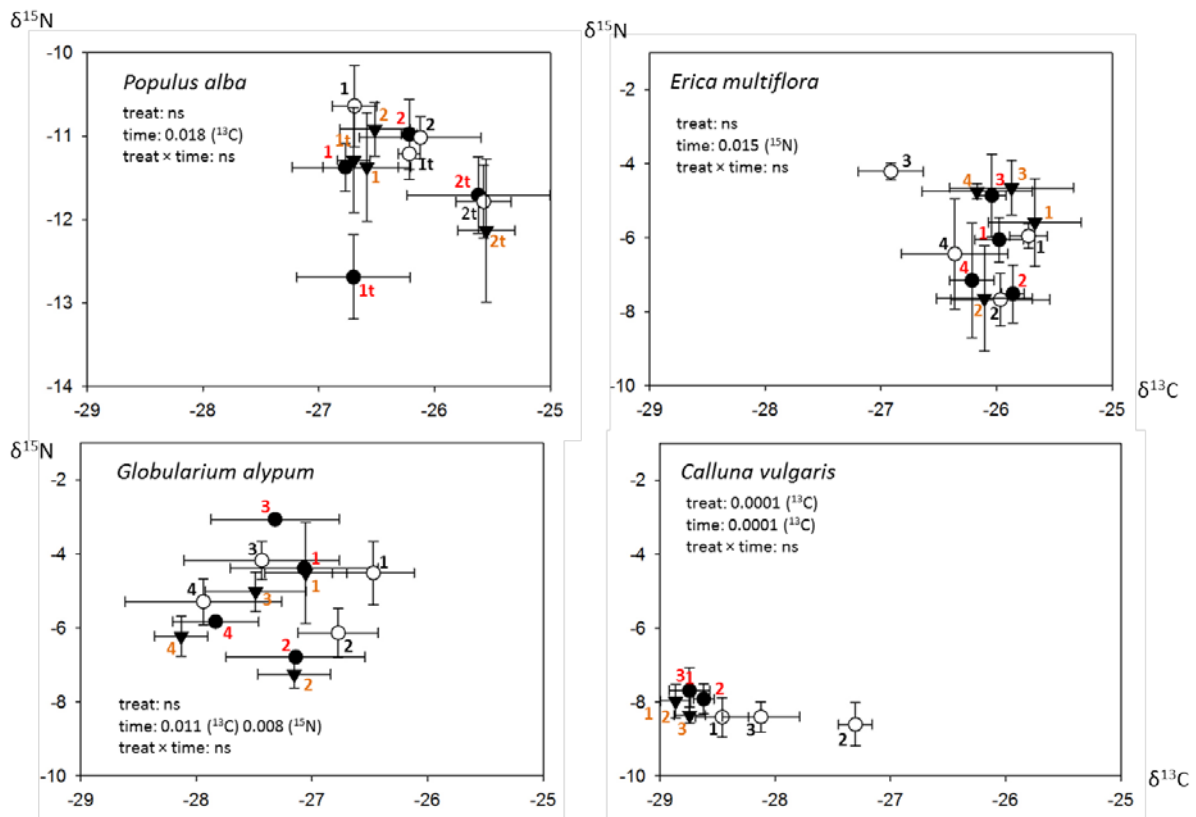
901 **Table 1.** Suggestions and advice to consider when applying isotopic methods for the study of
902 carbon fluxes in the plant-soil system.

| Method | Expenses (cost) | Advice (do's and don'ts) | Before you start | Data analysis hint | Time spent |
|--|---|--|--|--|---|
| Bomb-C (natural ^{14}C abundance) | High (AMS analysis); Equipment for CO_2 sampling is relatively cheap (closed chambers, carbon free pump, batteries, and molecular sieve system). An IRGA is also required. | <ul style="list-style-type: none"> - Avoid materials and labs with possible ^{14}C contamination - If soil CO_2 is to be analysed in the field, long incubation times are required to get sufficient CO_2 for AMS analysis (typically >1 ml). - Think carefully about the soil depths to be analysed, and take the sample consistently. ^{14}C signatures might vary strongly along few cm in the soil. - If bulk soil ^{14}C is to be analysed, try to remove the roots as much as possible, because of their contrasted ^{14}C signature. | <ul style="list-style-type: none"> - Discuss your experimental setup and objectives with the Radiocarbon facility staff. - If you are not sure about potential ^{14}C contamination in your lab, use another lab or make a swipe test. - Plan carefully the minimum number of samples required, as AMS analysis are expensive. - Make previous trials to assess the incubation times required to get a sufficient CO_2 sample - Go through the whole process of sample preparation with a trial sample. | - Discuss your results with the Radiocarbon facility staff. | - Processing time depends on the type of sample, although is usually low; determination by AMS may take several months depending on the facility. |
| In situ ^{13}C pulse-labelling | ^{13}C - enriched compounds used for labelling and as standards are usually expensive; ^{13}C determination by IRMS is much cheaper than ^{14}C by AMS, although more expensive than ^{14}C by liquid scintillation (^{14}C labelling). | <ul style="list-style-type: none"> - Consider the target pools to be analysed and plan your doses consequently. Take the potential dilution of the label by the unlabelled root system or soil carbon pool into account. - Think about the trade-off between faster and stronger or weaker and longer ^{13}C pulses. - If your study requires a high ^{13}C enrichment, mind the potential risk of contaminating the site. - Avoid above ambient CO_2 concentrations in the chamber. - If you need to monitor CO_2 during your pulse, remember that IRGAs are rather insensitive to $^{13}\text{CO}_2$. - High sampling frequency immediately after the pulse application is recommended. | <ul style="list-style-type: none"> - Test your chamber and tubing materials for adsorption / desorption effects, and ensure these are without carbon content (use PTFE (Teflon) tape, not gluing paper-based). - Make a previous trial if possible and go through the whole process of sample preparation. | Report the label addition per area: $\text{g } ^{13}\text{C m}^{-2}$. | <ul style="list-style-type: none"> - Pulse labelling experiments are usually short, but intensive. High sampling frequency after the pulse is very time-consuming. - Sample processing depends on the type of sample and number of replicates. - Experiments requiring root washing or microbial compound extraction are time consuming. |
| Natural abundance of isotopes (^{13}C and ^{15}N) | Rather cheap IRMS analysis. | <ul style="list-style-type: none"> - Make sure the history of sampling site is known (previous labelling experiments?) | <ul style="list-style-type: none"> - Avoid sample contamination. - Be aware that FACE can dilute the isotopic signal, most CO_2 enriched systems use ^{13}C depleted sources, | - Make sure the experiment is feasible with sufficient $\delta^{13}\text{C}$ shift and fractionation expected to be strong enough to | <ul style="list-style-type: none"> - Sampling time and grinding / weighing of sample. - Analysis usually done at dedicated |

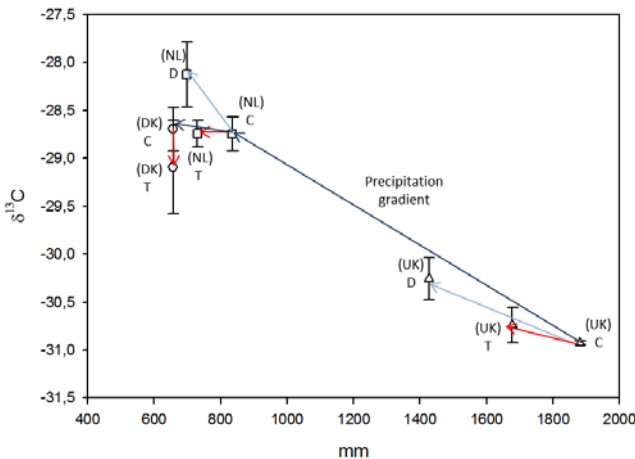
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|--|---|---|--|--|---|
| | | | because this is cheaper. | measure, base this on known discrimination at certain points in the carbon and nutrient cycle. | natural abundance facility. |
| ¹⁴C-substrates | Analysis of the trapped ¹⁴ C-CO ₂ is relatively cheap. | - High risk of contaminating lab equipment. | - You need to work in a dedicated ¹⁴ C lab safely away from the natural abundance facility. | | - Continue sampling until decline in emission is level, this ensures better model fit. |
| ¹³C-injection <i>in situ</i> | IRMS of dry matter plant material and soil cheap. The GC-c-MS of PLFAs for determination of ¹³ C-enrichment of individual PLFAs requires a specialised lab. | - Contamination risk of ¹³ C leaching is present, but smaller to our judgement than from ¹³ C-CO ₂ experiments. - Do not use areas dedicated to natural abundance work. | - Labelling intended for soil microbial components is more intense from ¹³ C liquid substrate <i>in-situ</i> injection than from ¹³ C-CO ₂ pulse labelling. | | - Soil sampling is destructive, consider to have several parallel plots to harvest an undisturbed plot at each sampling event. - Sample handling from field work until the extraction takes a few days so plan only one sampling event per week if possible. |

Box 1. Isotopic signal of plant leaf responses to precipitation

A



B

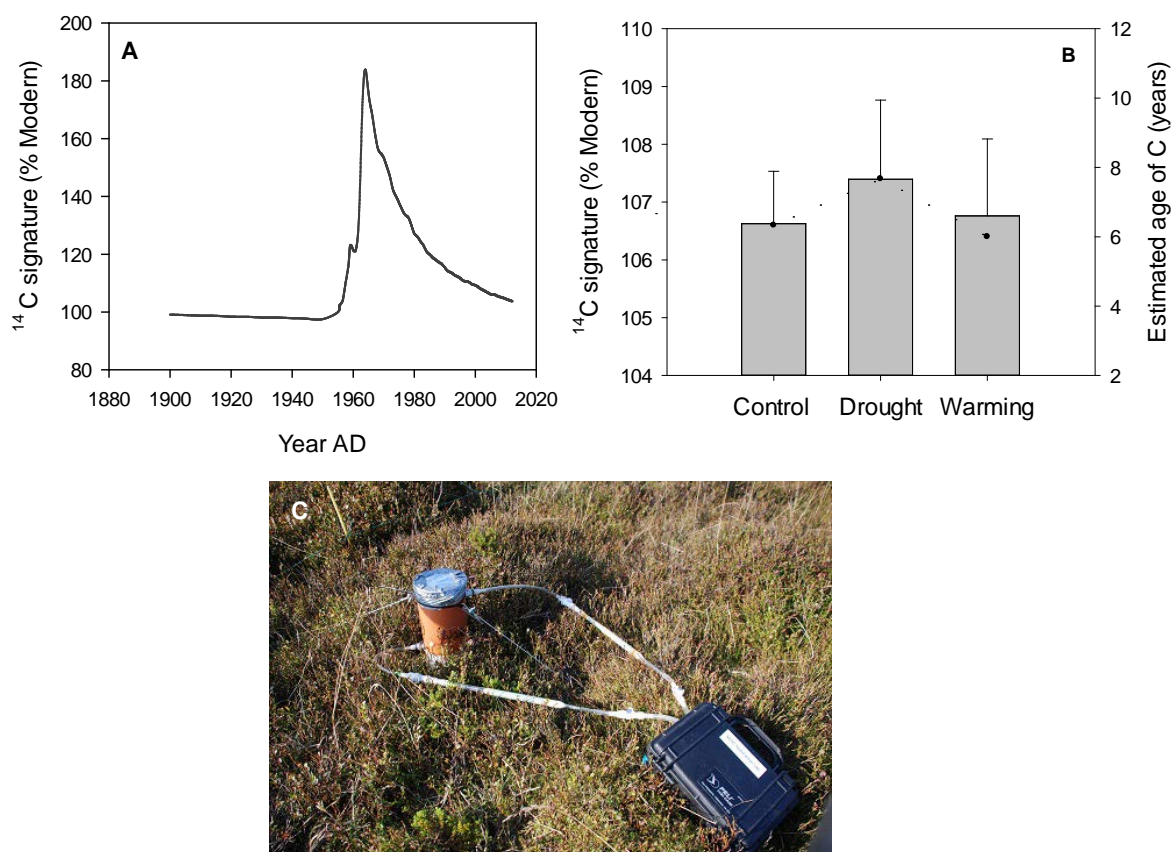


Stable isotopes in aboveground plant material: $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from isotopic ratio mass spectrometry (IRMS) analysis of solid samples. **A**: Leaves and twigs (t) from *P. alba* (HU), *E. multiflora* L. (SP), *G. alypum* L. (SP) and *C. vulgaris* (NL); filled circle ● is control, open circle ○ is drought treatment, ▼ is warming treatment. P-values indicate effects of treatment, year, and the interaction of these factors on ^{13}C or ^{15}N , analysed by two-way ANOVA; ns is non-

914 significant effect. Number indicates year (2001=1, 2002=2, 2003=3 or 2004=4). Species (site)
915 differences and annual differences are stronger than treatment effects. **B:** $\delta^{13}\text{C}$ of *C. vulgaris*
916 versus annual precipitation of the previous year. Within each site, leaf $\delta^{13}\text{C}$ was higher in the
917 drought treatment in comparison to control. Across sites, plants from drier sites (lower
918 precipitation) show higher leaf $\delta^{13}\text{C}$ values. The response to the drought treatment is the same
919 as moving to a drier site.

920

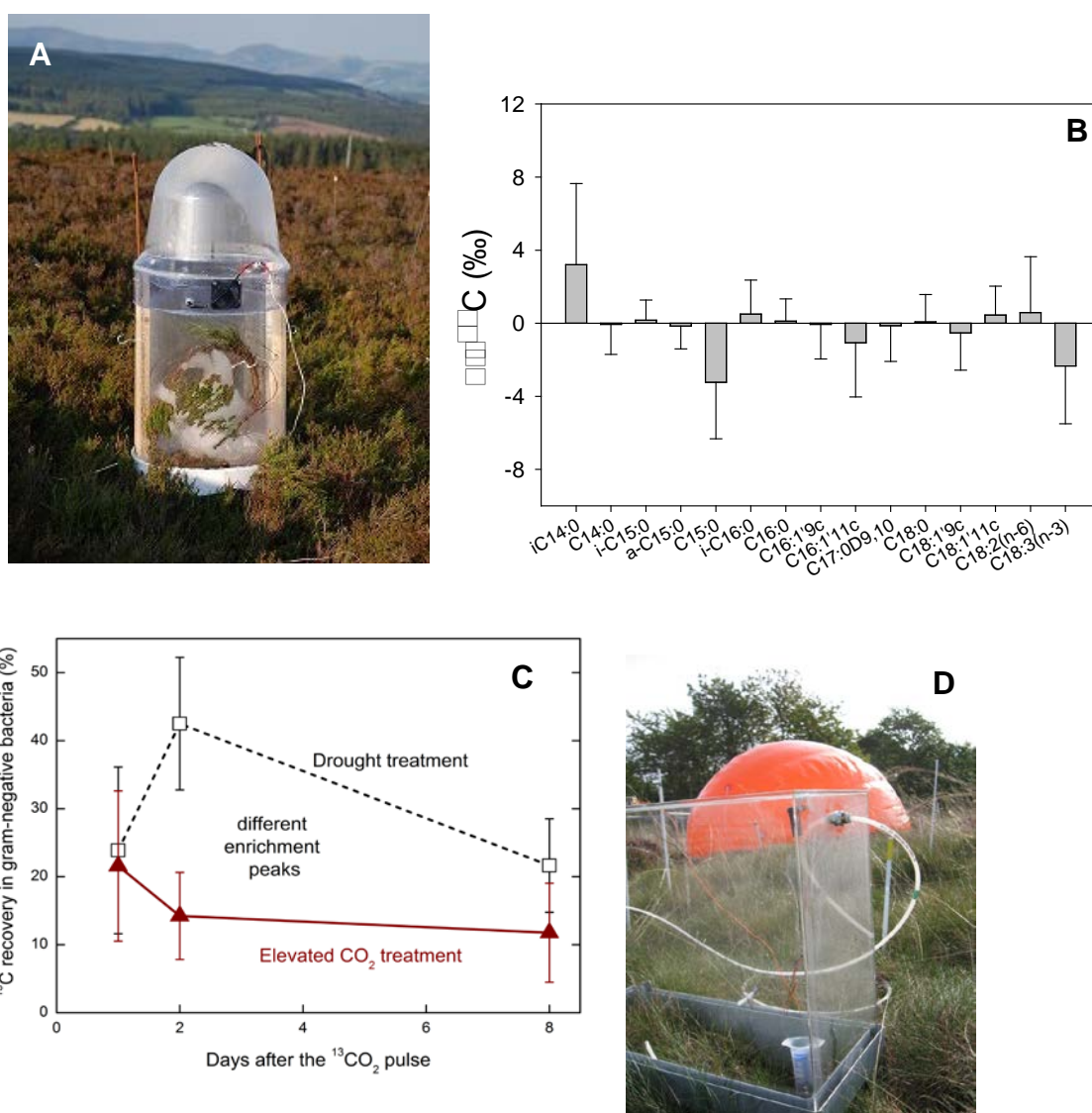
Box 2. Impact of warming and drought on the ^{14}C signature of soil respiration



Closed dark respiration chambers and a molecular sieve sampling system was used in the field (UK-PK) to collect CO_2 of the soil efflux for the analysis of its ^{14}C signature by Accelerator Mass Spectrometry (AMS). **A:** Recent and older carbon sources have contrasting radiocarbon (^{14}C) signatures as a result of the nuclear bomb tests in the atmosphere during the 1950/60s. These tests led to a global increase in the ^{14}C content in the atmospheric CO_2 , which reached its maximum in $\sim\text{AD}1963$. The unit for ^{14}C signature (% Modern) is a measurement of the deviation of the $^{14}\text{C}/^{12}\text{C}$ ratio of a sample from the "Modern" standard, which is defined as 95% of the radiocarbon concentration (in AD 1950) of a reference material (NBS Oxalic Acid I, SRM 4990B), adjusted to a $\delta^{13}\text{C}$ reference value of -19‰ . **B:** The ^{14}C signature of the soil efflux measured at the site (bars, left axis) was highly heterogeneous (ranging from 105.49 to 110.13 % Modern; values of $> 100\%$ Modern suggest that a substantial component (and potentially all) of the carbon was trapped by photosynthesis during the post-bomb era i.e. since $\sim\text{AD} 1955$), with no significant effect of the warming treatment, and a trend towards the release

of older carbon in the drought plots. On average, the carbon being released from the plots had been fixed from the atmosphere between six and eight years earlier (line, right axis). Incubations confirmed that the carbon respired by roots (mostly of *V. myrtillus* L.) was recently fixed (similar ^{14}C signature as the atmosphere at the time of sampling), while the carbon released from root-free soil samples (heterotrophic component) showed a variable range of ages, with substantial components of pre-bomb carbon (carbon fixed before AD 1955). **C:** Detail of a closed static chamber used to collect CO_2 from the soil efflux. Air is pumped in a closed loop from the chamber through a quartz glass cartridge containing a zeolite molecular sieve, which traps the CO_2 allowing it to be returned to the laboratory, recovered by heating, and analysed by AMS.

Box 3. Analysing rhizodeposit utilisation by microbes in the field



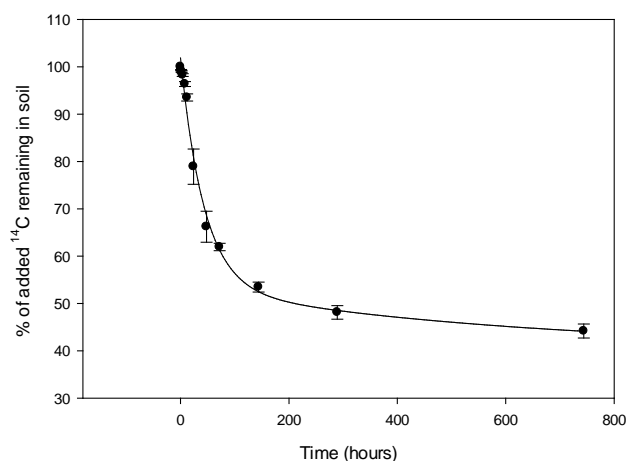
In-situ pulse-labelling experiments adding ^{13}C - CO_2 to closed transparent chambers were used to study the translocation of the recently-fixed carbon belowground and the rhizodeposition utilization by microbes, e.g. by measuring ^{13}C incorporation into microbial biomarkers. **A:** At the Clocaenog site (UK-CL) this technique was applied along a peat layer gradient. Repeated pulses of ^{13}C - CO_2 were applied during eight hours to *C. vulgaris* using sealed domes attached to a core inserted into the ground. **B:** The incorporation of ^{13}C into soil microbial PLFAs was analysed. Despite a high applied dose of ^{13}C (99 atom %), the dilution of the tracer within the large pool of unlabelled root biomass was remarkable, and as a consequence most of the analysed PLFAs showed no ^{13}C enrichment. **C:** ^{13}C recovery in Gram negative bacteria after a ^{13}C - CO_2 pulse at the Brandbjerg site (DK-BRA). The enrichment pattern in PLFAs attributed to Gram negative bacteria in soils exposed to drought and elevated CO_2 concentration (+120

976 ppm) for 8 years show different carbon utilization patterns and magnitudes under imposed
977 climatic conditions implying changed carbon cycle dynamics. **D:** Flow-through pulse-labelling
978 equipment showing the gas reservoir containing ^{13}C -CO₂ for up to eight hours of labelling
979 connected to transparent Plexiglas chambers via tubing.

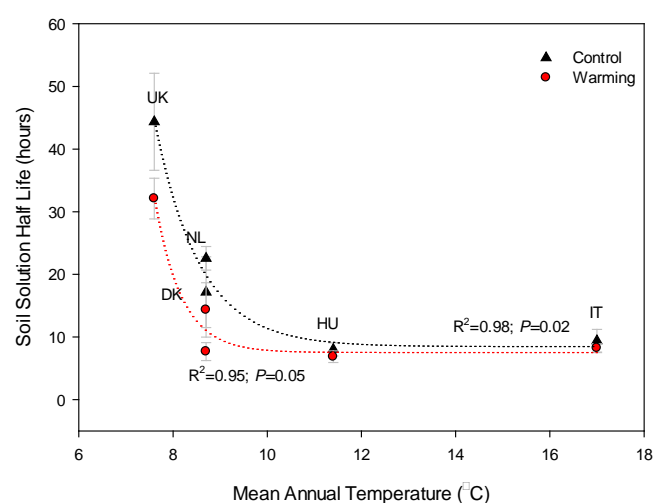
980

Box 4. Exponential decay kinetics for $^{14}\text{CO}_2$ evolution during microbial ^{14}C substrate mineralisation

A



B



The catabolic utilisation profile, turnover and pool allocation of low molecular weight (LMW) carbon substrates was determined in soils collected across the experimental network. A selection of sixteen ^{14}C labelled amino acids and sugars varying in structural complexity and recalcitrance were used in a multiple substrate induced respiration (SIR) assay on soil. Evolved CO_2 was collected using NaOH traps and absorbed $^{14}\text{CO}_2$ was measured with a scintillation counter (Perkin Elmer). The rate at which radiolabelled substrates were metabolised by the microbial community was used to determine microbial uptake kinetics and turnover in the

absence of plants (no autotrophic input). A double term first order decay model with an asymptote described our data best;

$$f = y_0 + a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \quad \text{eqn. 1.}$$

where f describes the amount of ^{14}C -labelled substrate or metabolites remaining in the soil at time t , the asymptote y_0 explains the ^{14}C labelled material adsorbed to unavailable soil complexes or metabolites partitioned into unavailable recalcitrant material, the exponential coefficient k_1 describes the initial rapid uptake and respiration of ^{14}C labelled substrate by the microbial community immediately following carbon substrate application, and k_2 describes a slower secondary mineralisation phase which we ascribed to immobilisation in microbial biomass and transformation of organic metabolites (microbial turnover). The terms a_1 and a_2 relate to the proportion of ^{14}C labelled substrate that is associated with each exponential coefficient at time (t).

The mean residence time (MRT) or substrate half-life ($t_{1/2}$) can be calculated according to

$$t_{1/2} = \frac{\ln(2)}{k_n} \quad \text{eqn. 2.}$$

The turnover of each pool can then be calculated as the inverse of the MRT (1/MRT).

A: For substrate mineralisation the equation: $y = 40.3766 + 48.3216^{0.0230x} + 13.1812^{0.0017x}$ fitted the data with an r^2 of 0.99. Using the coefficients (k_n) from the fitted equation, the half-life of the substrate can be calculated using eqn. 2. Substrate half-life was in the first phase (soil solution uptake) 30 h, and the second slower phase (microbial turnover) 408 h. Approximately 40 % of the substrate was immobilised in the soil, 48.3 % respired during the first phase, and 13.2 % respired during the slower second phase. **B:** Half-life of the substrate in the soil solution versus mean annual temperature, in control (triangle) and warming (circle) treatments, data points are mean \pm SE ($n=3$). Warming treatment and relative warmer site, simply increases the catabolic utilisation of labile LMW-carbon until a threshold mean annual temperature of 11.5 °C.